Phenoloxidase in the Armyworm, Leucania separata Walker

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7. アワヨトウのフェノールオキシダーゼ

池本 始（東京都立アイソトープ総合研究所，東京都世田谷区） 47. 1. 31 受理

アワヨトウ幼虫の体液フェノールオキシダーゼはモノフェノールオキシダーゼとしての基質特異性をしめし，5.6から8.0にかけて至適 pH がみられた．銅酵素の阻害剤で活性が抑制されるので銅蛋白質とおもわれる．淡色型幼虫（単独飼育）の体液を酵素液に用いるとチロシンを酸化するとき lag 期をしめしたが，黒色型幼虫（高密度飼育）では lag 期はみられなかった．原因の一つとして黒色型幼虫の体液は淡色型幼虫の体液に比較してドーパ量が多いことが考えられる．

黒色型幼虫は淡色型幼虫よりも体液，皮膚ともにつよいフェノールオキシダーゼ活性をしめした．つよいフェノールオキシダーゼ活性がアワヨトウの相変異にともなう黒化現象に関与しているとおもわれる．なお，体液フェノールオキシダーゼは蛹化のとき，いちじるしくつよい活性をしめしたが，これは蛹化のタンニング反応に関与しているとおもわれる．蛹化直後の皮膚フェノールオキシダーゼ活性はほとんどみられなかったが，皮膚では不溶性フェノールオキシダーゼがタンニング反応に関与しているものと推定される．

The larvae of the armyworm, *Leucania separata* show a greenish yellow or brown colour when reared in isolation. But those reared in high density show a fine velvety black in their dorsal region[6]. The black pigment of the larval integument is reported to be a kind of indole melanins[3].

It is well known that the phenoloxidase catalizes the production of quinones which are responsible for the hardening and darkening of the integument of insects after their molting and pupation.

In the present report, substrate specificity and some other characteristics of the armyworm phenoloxidase were investigated by using the haemolymph of the gregarious black larvae. Furthermore, phenoloxidase activities were compared between the isolated type and the high density type of the armyworm over several stages.

* This work was read at the meeting of the Tokai branch, Japanese society of applied entomology and zoology in Gifu, July, 1967.

Material and Methods

Insects

The larvae used in this study were reared in the laboratory on the leaves of cornplants by the method of Ikemoto[2].

Preparation of enzyme solution

Enzyme preparations were made as follows: Haemolymph was collected in cold tube to protect haemolymph from melanization. Samples collected from 15 to 30 individuals were mixed in order to obtain at least 0.8m*l* of haemolymph for each experiment, and 0.6m*l* of haemolymph was diluted to 4m*l* with M/15 phosphate buffer (pH 7.0). The diluted haemolymph was used as an enzyme solution.

Dorsal region of the abdominal integument was dissected out from the remainder of the body, and washed with distilled water. The integuments of about seven animals were pooled together, and homogenized with 15 volume of M/15 phosphate buffer (pH 7.0), and then after centrifuging, supernatant liquid of the homogena-

Table 1. pH dependence of the oxidation of catechol by armyworm phenoloxidase

pH	4.0	5.4	5.6	6.0	6.4	6.8	7.0	8.0	9.0	9.5	10.4
Relative activity	0	0.75	1.11	1.03	1.08	1.08	1.0	1.14	0.54	0.26	0.14

Haemolymph of the gregarious black larvae was used as enzyme solution. Acetate, citrate, phosphate, veronal and carbonate buffers were used for pH ranges of 4.0-5.4, 5.4-6.0, 6.0-7.0, 8.0-9.0, and 9.5-10.4, respectively.

tes were used as an enzyme solution. This experimental procedure was carried out at 3°C.

Measurement of phenoloxidase activity

The activity of phenoloxidase was estimated manometrically at 28°C. The reaction system used was 0.75m*l* of phosphate buffer (M/15, pH 7.0), 0.45m*l* of distilled water and 0.5m*l* of substrate solution in the main vessel and 0.3m*l* of enzyme solution in the side arm and 0.2m*l* of 10% KOH solution in the center well. After temperature equilibration was reached at 28°C, the reaction was initiated by introducing the enzyme solution from the side arm into the main vessel chamber. Oxygen uptake was observed at 5 minute intervals. The enzyme activity was measured from the reading of the first 10 minutes of the time course of oxygen uptake and expressed as μl.

In inhibition experiment, 0.45m*l* of distilled water in the reaction mixture was replaced by an inhibitor.

Results and Discussion

Nature of haemolymph phenoloxidase

Using catechol as substrate, the effect of pH on the enzyme activity was studied. The results are given in Table 1. Phenoloxidase of haemolymph shows a plateau of activity ranging from pH 5.6 to 8.0. This is followed by a marked decline over pH 9.0 and 10.4. Ohnishi showed that phenoloxidase activity of *Drosophila virilis* appeares to be of no significant difference ranging from pH 5.5 to 7.0[11].

Waku and Iwao showed that armyworm phe-

Table 2. Phenoloxidase activity to different substrates (μl)*

Substrate	Activity (a)	Activity on catechol 0.25×10^{-1}M(b),	0.25×10^{-2}M(c)	Ratio a/b(c)×100
o-Cresol	0			
o-Cresol+ Catechol 0.25×10^{-2}M	15.0	61.6	19.0	24 (79)
m-Cresol	0			
p-Cresol	56.5	57.5		98
Guaiacol	0			
Guaiacol+ Catechol 0.25×10^{-2}M	16.9	58.3	19.6	37 (86)
Tyrosine	21.0	57.5	18.2	(115)
Resorcin	0			
Resorcin+ Catechol 0.25×10^{-2}M	2.7	58.1	17.6	5 (16)
Dopa	57.3	58.0		99
Hydroquinone	0			
Hydroquinone+ Catechol 0.25×10^{-2}M	64.3	61.4	18.1	105(356)
Pyrogallol	24.7	57.5		35
Phloroglucinol	0			
Phloroglucinol+ Catechol 0.25×10^{-2}M	14.4	58.0	16.1	25 (89)
Hydroxyhydroquinone	117.5	57.5		204
Dimethyl-*p*-phenylenediamine	2.0			

* Haemolymph from the gregarious black larvae was used as enzyme solution. Substrate concentration, 0.25×10^{-1}M (except for tyrosine of 0.25×10^{-2}M).

noloxidase catalizes aerobic oxidation of diphenol, catechol[13]. As shown in Table 2, it was found that the enzyme in the haemolymph of the armyworm oxidizes *o*-diphenol (dopa, catechol, and pyrogallol) and *p*-monosubstitutedphenols (*p*-cresol and tyrosine). Oxygen uptake was not observed with *o*-cresol, guaiacol, hydroquinone, phloroglucinol and dimethyl-*p*-phenylenediamine. Hydroquinone could be oxidized indirectly, however, by adding a small amount of catechol to the reaction system, as in the case of *Drosophila* phenoloxidase[11].

It is well known that there is a lag phase in the early stage of aerobic oxidation of monophenols, i. e., *p*-cresol and tyrosine. As shown in Figure 1, when haemolymph from isolated pale larvae was used as enzyme solution, there was a lag period for 8 minutes, and thereafter the rate of oxygen uptake rose linearly in the oxidation of tyrosine. Enzyme preparation from the gregarious black larvae, however, exhibited

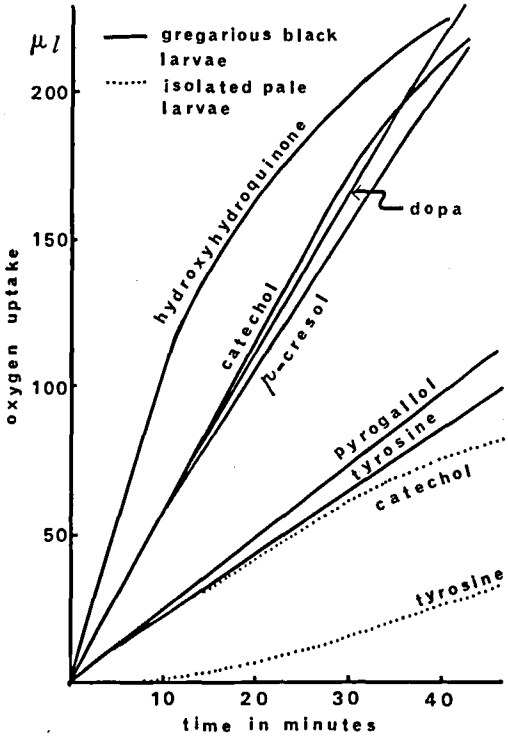

Fig. 1. Time course of oxygen uptake by haemolymph phenoloxidase.
Substrate concentration, 0.25×10^{-1}M (except for tyrosine of 0.25×10^{-2}M).

no lag period in the oxidation of monophenols.

Nakamura and Sho reported that catechol and dopa or reducing agents such as hydroquinone, ferrocyanide and ascorbic acid were able to shorten the lag period of monophenol oxidation in the silkworm phenoloxidase[8]. Ikemoto showed that the content of dopa in the larval haemolymph from crowded culture is higher than that in the larval haemolymph from isolated culture[2]. The phenomenone mentioned above may be explained partly by the occurrence of dopa in a considerable amount in haemolymph of the gregarious black larvae. When catechol and hydroxyhydroquinone were used as substrates, the rates of oxygen uptake fall with the lapse of time. Similar change in oxygen uptake was observed in the case of the silkworm haemolymph phenoloxidase[4].

Effects of various inhibitors on phenoloxidase activity are summarized in Table 3. From these data, it was found that activity was inhibited by any agents which combine with heavy metals,

Table 3. Effects of inhibitors on phenoloxidase activity

Inhibitor	Final molar concentration	% Inhibition
Na diethylthiocarbamate	10^{-3}	100
	10^{-4}	5.2
Thiourea	10^{-2}	100
	10^{-3}	67
	10^{-4}	0
Monoiodoacetic acid	10^{-2}	18.9
	10^{-3}	8.5
p-nitrophenol	1.5×10^{-2}	100
	0.25×10^{-2}	0
KCN	10^{-3}	95
Sodium azide	10^{-2}	47.9
	10^{-3}	0

Larval haemolymph from crowded culture was used as enzyme solution.

especially with copper. Therefore, it is reasonable to assume that armyworm enzyme, like all other phenoloxidase, is a copper protein.

Phenoloxidase activity in haemolymph and integument of the armyworm over several stages

Figure 2 shows the changes in phenoloxidase activities in haemolymph of both types during the period from the late stage of the fifth instar

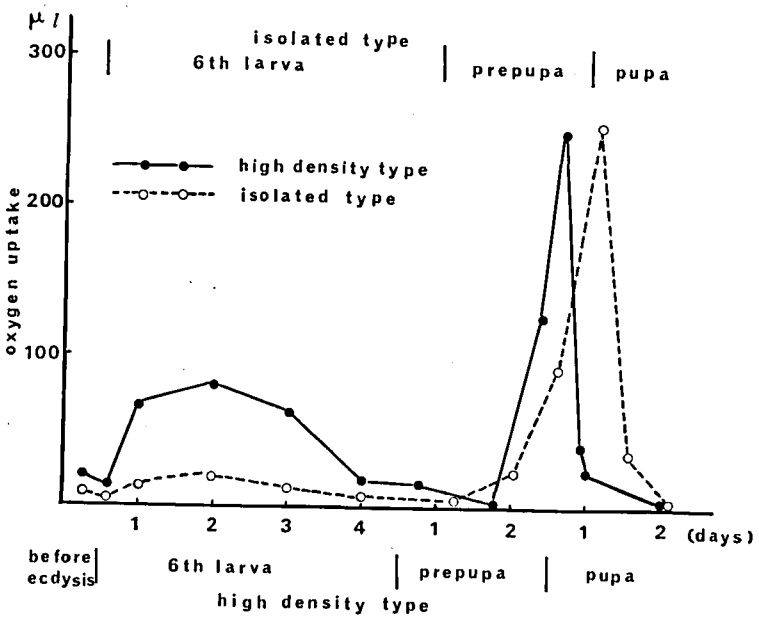

Fig. 2. Changes in phenoloxidase activity of the armyworm haemolymph at different stages.
Catechol was used as substrate.

to the beginning of the pupal stage.

Shortly before pupation the activity of each type increases rapidly and reaches maximum at the time of the initiation of pigmentation and hardening of the pupae, then decreases quickly and becomes almost nill, as in the case of *Bombyx mori* and *Prodenia blitura*[5,9,12]. The above measurement suggests an intimate relation between phenoloxidase activity and pupation.

Haemolymph of the gregarious black larvae showed higher phenoloxidase activity than that of solitary pale larvae. But the difference was not recognized in phenoloxidase activity of haemolymph between the isolated type and the high density type during the period from the prepupal to pupal stage.

Figure 3 shows the changes in phenoloxidase activities in the integument of both types over several stages.

Phenoloxidase activity in the integument of each type becomes high at the middle of the sixth instar period, then decreases untill maturate larval stage, and then increases abruptly reaching again at a high level just after prepupation, followed by a decease to the lowest level just after prepupation, followed by a decease to the lowest level just after pupation. The integuments of the gregarious black larvae showed higher phenoloxidase activity than those of the isolated pale larvae. It is interesting that phenoloxidase activity of the integumental extract becomes almost nill just at pupation. The result obtained is consistent with Kawase's[7] observation on the silkworm. It is not necessary to infer from this that the autoxidation of the tyrosine metabolites plays some significant roles in the hardening and darkening which take place in the integument during pupation period. Because the water-insoluble enzyme may probably be responsible for the hardening and darkening of the integument after pupation as in the case of the silkworm[14].

The fact that phenoloxidase activities in both haemolymph and integument of the black larvae are higher than those of the isolated pale larvae suggests that phenoloxidase may be related to the difference of the ability of melanin formation. The author ascertained the followings: (i) Black pigment of the larvae produced under crowded condition is a kind of indole melanins[3], (ii) Free tyrosine is the main substrate of the haemolymph

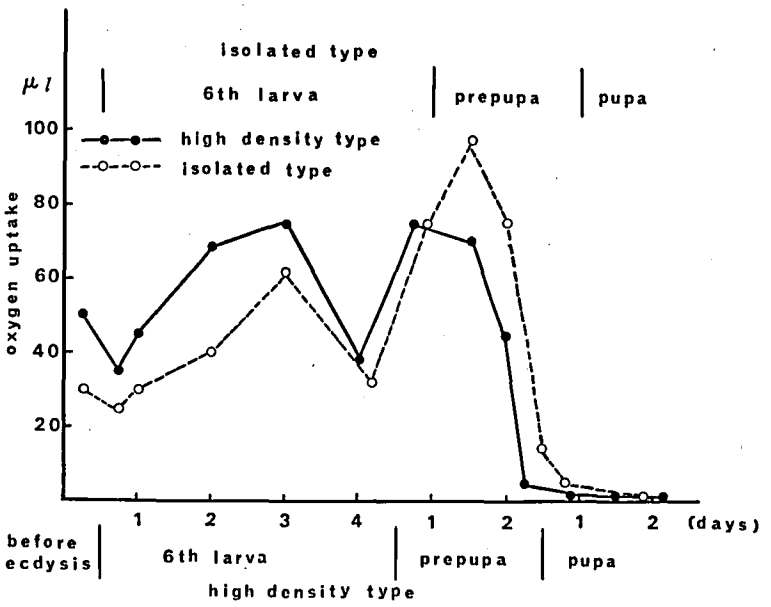

Fig. 3. Changes in phenoloxidase activity of the armyworm integument at different stages.
Catechol was used as substrate.

phenoloxidase, (iii) The content of dopa in the larval haemolymph from the crowded culture is higher than that in the larval haemolymph from the isolated culture[2]. And it is believed that indole melanins are synthesized by the action of phenoloxidase on tyrosine, the first product formed being dopa which undergoes further oxidation.

From these considerations, it seems likely that crowded culture results in an increased amount of dopa as a result of the stimulation of phenoloxidase activity in haemolymph, more dopa formed in haemolymph pass into the integument and then more indole melanin is produced from a considerable amount of dopa by the increased activity of phenoloxidase through some intermediates in the larval integument. The indole melanin formed in this way is responsible for the blackened coloration relating to larval density. In silkworm mutations, the formation of larval dark integument is due to the difference of phenoloxidase activity in the integument rather than that of the haemolymph phenoloxidase[1].

The possibility remains that the larval phenoloxidase of the armyworm occurs in similar amount in the two types but that the phenoloxidase of the isolated pale larvae is inhibited by the subtance(s) like the sulfhydryl-containing compound(s) which occurs in haemolymph and the integument of the isolated pale larvae. It has been reported that in locust the phenoloxidase occurs as much in the green solitaria as in the black gregaria hoppers, and inhibitor(s) is present to a greater extent in the solitaria hoppers[10]. From this phenomenon, detailed experiments on these subjects can be performed in the future.

It has bees known that pupae ecdysis from both isolated and crowded culture do not differ in their colour, in spite of their remarkable colour difference appearing in the larval stage[6]. It is interesting in this connection that there is no difference in phenoloxidase activity between both types during the course of development from late prepupa to pupa.

Summary

1. Phenoloxidase of the armyworm haemolymph has the characteristics of typical monophenoloxidase.

2. Phenoloxidase activity in haemolymph increases significantly at the time of pupation.

3. When haemolymph from solitary pale larvae

was used as enzyme solution, there is a lag period in the oxidation of monophenol, tyrosine. On the other hand, haemolymph prepared from the gregarious black larvae shows no lag phase in the oxidation of monophenols.

4. The gregarious black larvae show a higher phenoloxidase activity in both haemolymph and integument than the isolated pale larvae.

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Studies on Pyrethroidal Compounds* Part II. Comparative Activity of Pyrethrins I, Pyrethrins II and Other Synthetic Pyrethroidal Compounds. Kosuke Tsuda, Yasuo Abe and Yoshio Fujita (Research Department, Pesticides Division, Sumitomo Chemical Co., Ltd., Takatsukasa, Takarazuka City, Hyogo, Japan.) Received February 22, 1972. *Botyu-Kagaku*, **37**, 48 1972.

8. ピレスロイド系化合物の研究 第2報 Pyrethrins I, II および数種合成ピレスロイドの効力比較 津田小亮，安部八洲男，藤田義雄（住友化学工業株式会社宝塚研究所農薬事業部研究部，兵庫県宝塚市高司4丁目） 47. 2. 22 受理.

ピレトリンエキスおよびそれから薄層クロマトグラフィーで分離精製した Pyrethrins I, II 並びに4種の合成ピレスロイド（アレスリン・テトラメスリン・レスメスリン・フラメスリン）の殺虫効力を比較し，ピレスロイド混合物の連合作用について検討した．その結果ピレトリンエキスの殺虫効力は Pyrethrins I と Pyrethrins II のみによるもので，前者は主に致死剤として後者はノックダウン剤として作用し両者間に僅かの協力作用が認められた．しかし，Pyrethrins II は線香の殺虫効力にはほとんど寄与しない．オイルスプレーテストではテトラメスリンは優れたノックダウン速効効果を示し，レスメスリンは高い致死効果を示した．更に両者間には協力作用がみられた．アレスリンに少量のレスメトリンを添加した線香の希薄煙はノックダウン効果において速効性と同時に効果の持続性が認められた．また，供試ピレスロイドに対して Lab-em-7-em 系（感受性）および 203 d系（ダイアジノン抵抗性）イエバエ間に明瞭な感受性差異は認められなかった．

Introduction

Pyrethrum extract has been widely used as an insecticide from ancient times due to its quick knock-down effect to insects and low toxicity to mammals. "Pyrethrins", the insecticidal principles, consist of six insecticidal esters; cinerin I (cin. I), jasmolin I (jas. I), pyrethrin I (pyr. I), cinerin II (cin. II), jasmolin II (jas. II), and pyrethrin II (pyr. II). Pyrethrin I and II are more toxic than the corresponding cinerin I and II to houseflies[1~5] and mustard beetles[6,7]. Pyrethrins I (a mixture of cin. I, jas. I, and pyr. I) are recovered in better yield than pyrethrins II (a mixture of cin. II, jas. II, and pyr. II) from smoke of mosquito coils[8]. Cinerin I and II are more stable than pyrethrin I and II to sunlight[9].

* Part I of this series appeared in Reference [20].